

WO 00/67591

4/PRTS

1

Rec'd PCT/PTO 06 NOV 2001  
PCT/GB00/01753  
10/009583

## 1 "PIGMENT"

2

3 This invention relates to a method of altering and  
4 improving the pigmentation of fish flesh. More  
5 specifically the invention discloses a method of  
6 enhancing the uptake of pigments by fish, such that  
7 there is a resultant increase in the level of  
8 pigmentation of the fish flesh.

9

10 An increase in market competition, coupled with the  
11 widespread availability of fish in supermarkets has led  
12 to an increase in the demand for, and quality of fish  
13 products.

14

15 Mass production of salmonids such as salmon and trout  
16 is required to meet current consumer demand which  
17 exceeds that which can be met by fish produced in a  
18 natural, wild environment.

19

20 Variations exist between fish produced naturally and  
21 those which are specifically farmed to meet consumer

WO 00/67591

PCT/GB00/01753

2

1 demand. One particular difference is a variation in  
2 the colour of the flesh of the fish.

3

4 The characteristic pink colour of salmonid flesh is a  
5 result of the deposition of naturally occurring  
6 carotenoid pigments. Obtaining pigmentation in farmed  
7 salmonids which is similar to that seen in wild salmon  
8 is a vital aspect of feed production. Currently fish  
9 feeds contain either or both of the main synthetic  
10 pigments which are commercially available; astaxanthin  
11 (Ax) and canthaxanthin (Cx). In several instances,  
12 pigment costs contribute to 10-15% of the total cost of  
13 fish feed production, compared to pigment flesh  
14 deposition efficiencies which rarely exceed 15%. Since  
15 fish feed comprises around 50% of the total production  
16 costs of farmed fish, 5-7.5% of overall fish production  
17 cost can be attributed to the cost of pigment.

18

19 Flesh colour is one of the main criteria used by the  
20 consumer when considering the purchase of salmonids and  
21 accordingly it is considered by the consumer that the  
22 stronger red colour of the flesh which is seen in wild  
23 fish is more desirable.

24

25 In an effort to achieve the flesh colour  
26 characteristics exhibited by wild fish, pigments are  
27 added to the feed given to farmed fish with the intent  
28 that the uptake, by ingestion of the pigment, will lead  
29 to an associated change in the colour of the flesh.

30

WO 00/67591

PCT/GB00/01753

- 3
- 1 Pigments are specifically selected such that their  
2 uptake will lead to the flesh becoming a red colour.  
3 Examples of pigments which induce this are  
4 canthaxanthin and astaxanthin.
- 5
- 6 Such processes are not limited to fish, as the  
7 modification of the colour of naturally produced  
8 foodstuffs is a current trend. The aesthetic appeal of  
9 the product to the end consumer is enhanced through  
10 modification of the feed ingredients to influence the  
11 characteristics of the final product, in particular the  
12 colour of the product.
- 13
- 14 An example of such a process currently known in the art  
15 is the alteration of the feed ingredients given to  
16 chickens and hens, such that the colour of the yolk of  
17 the eggs that are produced is modified from that of the  
18 natural colour. The result of this process is that the  
19 product has an increased aesthetic appeal which in turn  
20 leads to a greater desirability for consumer  
21 consumption.
- 22
- 23 It is desirable for the flesh of the fish to be altered  
24 to any specific requirement which may be set. One such  
25 method of altering the fish flesh colour would be  
26 through the introduction of pigments into the diet.
- 27
- 28 It is an object of the present invention to provide a  
29 method for improving the uptake of pigments which are  
30 provided in the diet to influence the colour of fish  
31 flesh.

CONFIDENTIAL DOCUMENT

WO 00/67591

PCT/GB00/01753

4  
1 According to the present invention there is provided, a  
2 method of enhancing the uptake of pigment by fish, the  
3 method comprising feeding fish with cholesterol.

4

5 Preferably fish are fed cholesterol and pigment.

6

7 Preferably the cholesterol and/or pigment will be a  
8 component of the fish feed.

9

10 Also preferably the cholesterol will be provided in the  
11 same medium as the pigment.

12

13 Preferably the cholesterol will be added to the fish  
14 feed at a level of between 0.1 to 5 percent.

15

16 Most preferably the cholesterol will be added to the  
17 feed at a level of between 1 to 3 percent.

18

19 Preferably, the pigment will lead to a change in flesh  
20 colour, plasma pigment levels and flesh pigment levels  
21 of fish.

22

23 Preferably the method can be used on Atlantic salmon,  
24 rainbow trout, other salmonid species, tropical fish.

25

26 Alternatively, the method may be used on any other fish  
27 species where the pigment colour of either the flesh or  
28 skin is important.

29

EPO EPO EPO EPO EPO EPO EPO EPO EPO

WO 00/67591

PCT/GB00/01753

5

1 The invention also provides fish feed comprising  
2 cholesterol and pigment.

3

4 The invention also provides the use of cholesterol to  
5 enhance uptake of pigment to fish flesh.

6

7 Deposition of carotenoids in the fish flesh occurs as a  
8 result of several processes; absorption of pigments in  
9 the digestive tract, transport of pigment in the blood,  
10 retention in the flesh and metabolism of carotenoids.

11 These processes are further detailed below;

12

13 1. **Absorption**

14

15 Pigment absorption across the intestinal wall,  
16 from the digestive tract to the blood is the  
17 initial phase in pigment retention by muscle in  
18 salmonids. Since carotenoids are lipid soluble  
19 they are most likely to be emulsified in a mixed  
20 micelle together with bile, other lipid  
21 components, prolipase and lipase during absorption  
22 across the gastrointestinal tract (Leger 1985).

23

24 The rate of pigment absorption to the blood,  
25 following ingestion is fairly slow, compared to  
26 the absorption of essential fatty acids and amino  
27 acids (Storebakken & No, 1992). Maximum plasma Ax  
28 and Cx levels occurred at 24 hours following the  
29 force feeding of rainbow trout with a 500mg dose  
30 of Ax (March et al 1990, Choubert et al 1987),

KODAK SAFETY FILM

WO 00/67591

PCT/GB00/01753

1                         6  
2       carotenoid levels first being detected at 3 hours  
3       following feeding.

4       2. **Blood Transport**

5  
6       Ax and Cx are largely transported by the high  
7       density lipoprotein fraction of plasma in immature  
8       rainbow trout (Choubert et al, 1992, 1994).  
9       Generally in immature fish, flesh is a major  
10      tissue for storing carotenoids (No and  
11      Storebakken, 1992).

12  
13     3. **Deposition/Flesh Retention**

14  
15     Deposition efficiency of dietary carotenoids in  
16     salmonid flesh is in the range 1-18% (Torrisen et  
17     al, 1989). Astaxanthin retention efficiency of  
18     Rainbow trout was found to be significantly higher  
19     than that for canthaxanthin; 11.4% and 7.1%  
20     respectively (Storebakken & Choubert 1991). Dose  
21     response studies show that the efficiency of  
22     deposition declines with increase in dietary level  
23     (50 mg/kg in Rainbow trout, Storebakken & No 1992;  
24     10 mg/kg in Rainbow trout, Crampton 1995).

25  
26     Differences in flesh retention efficiencies  
27     between species have been observed, and it is  
28     known that rainbow trout (RBT) pigment has a  
29     greater efficiency than Atlantic salmon (ATS).

30

WO 00/67591

PCT/GB00/01753

In the muscle of wild salmon (*Oncorhynchus keta*,  
*O. nerka* & *O. kisutch*) astaxanthin (90% in the  
free form) and canthaxanthin are bound to  
actomyosin, probably via weak hydrophobic bonds  
(Henmi et al 1987). Astaxanthin forms two  
hydrogen bonds per one  $\beta$  ionone ring, and combines  
more strongly than canthaxanthin, due to its  
hydroxyl group (see Henmi et al 1989). The  
actomyosin of salmon muscle can associate with  
many kinds of carotenoids and lipids, implying  
that specificity of binding sites is not a  
problem, with variation between molecule types  
relating to the bond strength (Henmi et al 1989).  
In the skin the majority of astaxanthin is found  
in the ester form (Torrisen et al 1989).

## 17.4 Metabolism

19 Carotenoids and their metabolites have been  
20 detected in the tissues of fish up to 96 hours  
21 following ingestion of a labelled meal (Guillou et  
22 al 1992). Schiedt et al (1989) found idoxanthin  
23 to be a metabolite of astaxanthin in ATS flesh -  
24 higher levels of idoxanthin were found in  
25 experimental fish in indoor tanks of farmed fish  
26 in open cages, which suggests that this may be  
27 stress related (Al-Khalifa & Simpson 1988).  
28 Metabolites of carotenoids are found mainly in the  
29 skin, but also in the flesh of sexually maturing  
30 fish (Hata & Hata 1975; Scheidt et al 1985).

WO 00/67591

PCT/GB00/01753

8

1 Schiedt et al (1985) evidenced that astaxanthin  
2 could be a precursor to vitamin A in vitamin A-  
3 depleted fish. Results of Al-Khalifa & Simpson  
4 (1988) showed that astaxanthin was converted to  
5 zeaxanthin, but in Vitamin A sufficient RBT it was  
6 not converted to Vitamin A<sub>1</sub> and A<sub>2</sub> although fish  
7 fed a diet lacking in vitamin A and carotenoids  
8 for 30 days and then force fed astaxanthin showed  
9 an increase in vitamin A.

10

11 This document suggests that the incorporation of a  
12 pigment into the diet, either in combination with the  
13 foodstuffs directly, or as a separate entity introduced  
14 into the diet such that it will enter the same  
15 metabolic pathways as other ingested and absorbed  
16 nutrients will also end up as a constituent of the  
17 flesh.

18

19 The pigment will lead to a change in the colour of the  
20 flesh into which it is incorporated.

21

22 The incorporation of the pigment into the flesh may not  
23 be efficient and this document identifies a method of  
24 enhancing such pigment uptake.

25

26 The benefits of a method by which the uptake of pigment  
27 by the fish is enhanced are wide-ranging and cover both  
28 biological and economical aspects.

29

EPO/EPO-EPO/EPD/PDT

WO 00/67591

PCT/GB00/01753

9

1 The addition of pigments such as astaxanthin and  
2 canthaxanthin can have a drastic economical effect on  
3 the cost of producing fish feed pellets, due to the  
4 expensive cost of the pigments. As such a more  
5 efficient mechanism of producing the effects of  
6 astaxanthin and canthaxanthin may lead to a reduction  
7 in the amount that needs to be added to the feed  
8 initially.

Some research has indicated that lipid levels improve pigment absorption for example Choubert et al (1991) found that digestibility of Cx was greatly improved when using a lipid rich diet (14% lipid/dry matter cf 4% lipid/dry matter). However, at commercially realistic levels of lipid (24-35%) no differences were found in flesh deposition efficiencies of RBT (Crampton, 1996 internal data).

18  
19 Bjerking et al (1997) found no significant effect of  
20 dietary protein sources (eg a fish meal against a full  
21 fat soyabean meal) in Atlantic salmon fed for 9.5  
22 months, on the amount of astaxanthin in the muscle or  
23 the visual colour score.

24  
25 A study of biological utilisation of carotenoids ( $\alpha$  and  
26  $\beta$ -carotene) in rats found that bioavailability of  
27 naturally occurring carotenoids was greater than the  
28 crystalline form (Tee et al 1996). In addition, Bierer  
29 et al (1995) found that in pre-ruminant calves higher  
30 serum levels of carotenoids were observed when given

WO 00/67591

PCT/GB00/01753

10  
1 commercial beadlet sources compared to crystalline  
2 sources.

3

4 A Patent Application in the name of Finnfeeds  
5 International Limited, (WO 9818345 A) claims that the  
6 absorption in fish, crustaceans and healthy poultry of  
7 pigments present in a non-viscous animal feed is  
8 promoted by the presence of a carbohydrase and protease  
9 enzyme.

10

11 In studies with young chickens Tyczkowski et al (1989)  
12 found that lipids, long chain saturated fatty acids  
13 (myristic, palmitic, stearic) and triglyceride,  
14 tristearin, promoted minimal absorption of lutein,  
15 whereas the short chain saturated lauric acid promoted  
16 the highest absorption. Screening trials have been  
17 conducted to try and identify enhancers of pigment  
18 uptake that may be added to the feed to improve  
19 pigmentation.

20

21 Cholesterol was tested as one of the enhancers, due to  
22 its properties as an auxiliary agent in uptake.  
23 Cholesterol is an important lipid in some membranes and  
24 the plasma membranes of eucaryotic cells are usually  
25 rich in cholesterol, this steroid also modulates the  
26 fluidity of eukaryotic membranes. Due to these  
27 properties cholesterol was identified as a substance  
28 with the potential to enhance pigment uptake.

29

2007 PCT/GB00/01753

WO 00/67591

PCT/GB00/01753

11

1 Cholesterol is added to the feedstuffs either by means  
2 of extruder or via flex coating with a level of  
3 addition between 0.5% and 5%. Natural levels of  
4 cholesterol in commercial fish feeds (derived mainly  
5 from fish oil) are up to approximately 0.5%.

6

7 In the same way that the pigmentation of salmonid  
8 flesh, eg Atlantic salmon, Coho salmon, Chinook salmon,  
9 Rainbow trout, Artic charr, is important to the  
10 consumer, the skin colour of tropical fishes is also an  
11 important quality characteristic. In this way the  
12 feedstuffs of the above-mentioned species could be  
13 modified in a similar way to effect the colour of flesh  
14 and skin, in addition to flesh pigment concentration  
15 (mg/kg).

16

17 A series of experiments are described below which look  
18 at whether there is an enhancement of pigment uptake in  
19 the plasma and flesh when the fish feed is supplemented  
20 with varying levels of cholesterol.

21

22 Experiment 1

23

24 Atlantic salmon of mean weight 120g, were fed for a  
25 period of 72 hours on one of two diets;

26

27 Diet 1: contains approximately 40ppm of canthaxanthin  
28 (Cx)

29 Diet 2: contains 40ppm canthaxanthin (Cx) plus 0.48%  
30 (total feed, 3% of the lipid coating phase) of  
31 cholesterol.

WO 00/67591

PCT/GB00/01753

- 12
- 1 Cx and cholesterol were added in the coating.  
 2  
 3 After feeding, fish were bled via the caudal vein,  
 4 using heparanised vacutainers, the blood samples were  
 5 centrifuged on site and the plasma removed and stored  
 6 frozen. Plasma pigment levels were analysed on HPLC.  
 7  
 8 Analysis results for the feeds are shown in Table 1.  
 9

10 TABLE 1 Cholesterol levels in feeds

11

Fish Feed	Cholesterol addition	% Cholesterol in feed
Uncoated feed	0	0.32
coated feed	0	0.27
coated feed	0.48%	0.53
coated feed	0	0.28
coated feed	0.48%	0.54

12

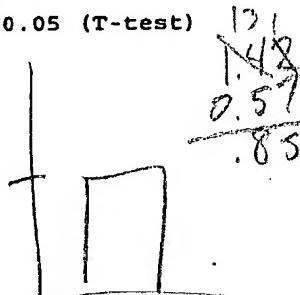
13

14 TABLE 2 Plasma results for the treatments

Replicate	Feed No.	Treatment	Cholesterol level % feed	Feed Cx mg/kg	Plasma Cx µg/ml mean (STD)
1	1	CR	0	40.51	0.94 (0.5)
	2				0.64 (0.4)
1	2	CR + Cholester-erol	0.48	45.67	1.42 (0.57)
	2				1.45 (0.96)

15

16 significant differences were observed p&lt;0.05 (T-test)



WO 00/67591

PCT/GB00/01753

WO 00/67591

PCT/GB00/01753

14

1 samples scored with respect to colour and later  
2 analysed for pigment.

3

4 The results of the experiments are further described  
5 with reference to the figures wherein;

6

7 Figure 1 shows the effect of feed cholesterol  
8 level on flesh pigment (Cx) concentration (mg/kg),  
9 with each point on the figure representing the  
10 mean value of each of the tanks,

11

12 Figure 2 shows the levels of the pigment  
13 canthaxanthin in fish flesh, when cholesterol is  
14 added to the feed effect of feed cholesterol level  
15 (mg/kg), wherein each point is the mean value of  
16 each tank (the 5 pooled analyses),

17

18 Figure 3 shows the effect of fed cholesterol level  
19 on fillet SalmoFan scores and,

20

21 Figure 4 shows the effect of feed cholesterol  
22 level on Minolta redness (a\* value).

23

24 Figure 1 shows that the plasma pigment levels show an  
25 increase which is correlated with an increase in  
26 dietary cholesterol to approximately 1-3%. Any further  
27 addition of cholesterol to the feed after this level  
28 shows a decline in pigment plasma concentration.  
29 Maximum canthaxanthin plasma level values were observed  
30 at 3.6 µg/ml (1% feed cholesterol added), compared to  
31 control values of 1.5-2 µg/ml.

RECORDED - INDEXED - SERIALIZED - FILED

WO 00/67591

PCT/GB00/01753

15

1     Figure 2 shows the effects on the levels of the pigment  
2     canthaxanthin in fish flesh, when cholesterol is added  
3     to the feed. Maximum flesh pigment levels of around  
4     4.3 mg/kg were observed in the group of fish fed  
5     canthaxanthin (which have a feed cholesterao level of  
6     1.3%), compared to levels of around 3 mg/kg in the  
7     control groups. In this size of Atlantic salmon,  
8     dietary cholesterol levels (1-4%) caused an increase in  
9     flesh pigment levels, this increase ranged from 0.4  
10    mg/kg to 1.3 mg/kg.

11

12    Astaxanthin flesh levels were 2.32 mg/kg for the  
13    control fish and 2.76 mg/kg for the fish with a 3.8%  
14    cholesterol supplement to their feed. Astaxanthin  
15    plasma levels were 0.62 µg/ml for the control and 0.65  
16    µg/ml for the fish whose feed was supplemented with  
17    3.8% cholesterol.

18

19    The effect of increasing the overall percentage of  
20    cholesterol in feed with respect to the resultant  
21    colour of the flesh is shown in Figure 3. The colour  
22    is scored using a Roche SalmoFan™ score. This is a  
23    tool used in the industry to score fish colour, which  
24    was developed by Hoffman la Roche Ltd. The test  
25    comprises a set of different coloured plastic mini  
26    sheets which combine to form a scale that ranges from  
27    20 (pale pink) - 34 (dark red), which are used to  
28    compare against the colour of the fish flesh and score  
29    them accordingly.

30

WO 00/67591

PCT/GB00/01753

16

1 Maximum SalmoFan scores were observed with the tanks of  
2 fish fed 1-2% cholesterol in the feeds. At higher feed  
3 cholesterol levels, a decrease in Roche SalmoFan™  
4 scores was observed (Figure 3). The difference in  
5 flesh colour shown by the fish fed diets which had been  
6 supplemented with between 1-2% cholesterol related to  
7 1-1.5 points advantage on the Roche SalmoFan™ test.

8

9 Further analysis of the flesh colour was carried out  
10 using the Minolta evaluation technique. Minolta redness  
11 values are shown in Figure 2. The Minolta is a  
12 tristimulus colorimeter (Minolta Chroma Meter CR300,  
13 Minolta, Japan) which has an 8mm head and a D65 light  
14 source. Readings were given for Lightness ( $L^*$ ),  
15 Redness ( $a^*$ ) and yellowness ( $b^*$ ), the "L a b" system  
16 according to International Commission on Illumination  
17 (CIE, 1986). Maximum redness values were observed in  
18 the fish fed which been supplemented with between 1-2%  
19 of cholesterol, although the pattern was not as clear  
20 as that exhibited by the results of the SalmoFan™  
21 scoring system.

22

23 In conclusion, although the experiments described  
24 herein show that the addition of any amount of  
25 cholesterol to a fish feed at the level of 0 to 5% can  
26 results in an increase in pigment levels in the plasma  
27 and flesh, the results indicate that the optimum uptake  
28 of pigment by the plasma and deposition in the flesh  
29 occurs when the feed contains a cholesterol level of  
30 between 1 to 3% of total feed weight.

תְּלִימָדָה

WO 00/67591

PCT/GB00/01753

17

1 Table 3:

Feed No	Cholesterol Feed Level (%)	Pigment Type	Dietary Pigment Conc (mg/kg)	Oil Source
		Control	Feed Level (%)	
1441	Control	0.473	Cantha	55.11
1442	Control	0.382	Cantha	44.51
1443	Control	0.305	Cantha	50.94
1444	1	1.258	Cantha	46.66
1445	2	2.186	Cantha	50.09
1446	3	3.142	Cantha	52.39
1661	4	4.001	Cantha	50.82
1662	4	3.936	Cantha	53.47
1663	4	3.802	Cantha	48.62
1664	Control	0.412	Asta	47.47
1665	4	3.803	Asta	44.86